Fibronectin binding proteins SpsD and SpsL both support invasion of canine epithelial cells by *Staphylococcus pseudintermedius*

Staphylococcus pseudintermedius internalization

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Abbreviations: BHI, brain heart infusion; BSA, bovine serum albumin; CFU, colony forming unit; CGP77675, 5,7-diphenyl-pyrrolo(2,3-d)pyrimidine; CPEK, canine progenitor epidermal keratinocytes; DMEM, high-glucose Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum; Fn, fibronectin; FnBPA/B, fibronectin binding protein A/B; GBD, gelatin-binding domain of fibronectin; HaCaT, cultured human keratinocyte; Hep-2, human epithelial cell line; LA, Luria agar; LB, Luria broth; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)]; N29, N-terminal fragment of fibronectin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 1-phenyl-1*h*-pyrazolo[3,4-*d*]pyrimidin-4-amine; PVL, panton-valentine leucocidin; RGD, serine-glycine aspartate peptide; RGE, serine-glycine-glutamic acid peptide; SpsD/SpsL, *Staphylococcus pseudintermedius* surface protein D/L.

ABSTRACT

In this study we investigated the cell wall-anchored fibronectin-binding proteins SpsD and SpsL from the canine commensal and pathogen *Staphylococcus pseudintermedius* for their role in promoting bacterial invasion into canine keratinocyte CPEK cells. Invasion was examined by the gentamicin protection assay and fluorescence microscopy. An *spsD/spsL* double mutant of strain ED99 had a dramatically reduced capacity to invade CPEK cell monolayers, while no difference in the invasion level was observed with single mutants. *L. lactis* transformed with plasmids expressing SpsD and SpsL promoted invasion showing that both proteins are important. Soluble fibronectin was required for invasion and an RGD-containing peptide orantibodies recognizing the integrin $\alpha_5\beta_1$ markedly reduced invasion, suggesting an important role for the integrin in this process. Src kinase inhibitors effectively blocked internalization suggesting a functional role forthe kinase in invasion. In order to identify the minimal fibronectin-binding region of SpsD and SpsL involved in the internalization process, recombinant fragments of both proteins were produced. The $SpsD_{520-846}$ and $SpsL_{538-823}$ regions harbouring the major fibronectin-binding sites inhibited *S. pseudintermedius* internalization. Finally, the effects of staphylococcal invasion on the integrity of different cell lines was examined. Because SpsD and SpsL are critical factors for adhesion and invasion, blocking these processes could provide a strategy forfuture approaches to treating infections.

Key words: *Staphylococcus*, adhesin, fibronectin, invasion, keratinocyte

INTRODUCTION

The Gram-positive bacterium *Staphylococcus pseudintermedius* is a common commensal of dogs (1, 2). The bacterium is also the most common pathogen associated with canine otitis externa and pyoderma as well as surgical wound infections and urinary tract infections (3). Sporadic cases of human infection have also been reported, including individuals exposed to colonized household pets (4-7). Genome sequence analysis (8, 9) indicated that *S. pseudintermedius* could encode many potential virulence factors including toxins, enzymes and surface proteins, some of which can promote adhesion of the bacterium to the surface of epithelial cells (10-13) and to components of the extracellular matrix (14,15)

Two cell wall-anchored surface proteins that are likely to be important in host tissue colonization and pathogenesis are SpsD and SpsL (Fig.1) (15). The primary translation product of SpsD from strain ED99 has an N-terminal secretory signal sequence and a C-terminal cell wall-anchoring domain (the sorting signal) comprising an LPXTG motif, a hydrophobic trans-membrane domain and a short sequence rich in positively charged residues. Residues at the N-terminus of SpsD are 40% identical to the fibrinogen-binding A domain of FnBPB from *S. aureus* and are predicted to fold into three subdomains N1, N2 and N3. This domain is followed by a connecting region C and a repeat region R. SpsL includes a signal sequence at the N terminus followed by an A domain with two IgG-like folds (N2 and N3), a domain containing seven tandem repeats with weak homology to the fibronectin binding repeats of FnBPA from *S. aureus* and a C-terminal sorting signal.

SpsD and SpsL mediate bacterial adherence to fibrinogen (15) and fibronectin (Fn) (15), while SpsD also binds to cytokeratin 10 and elastin (16). The binding site in fibrinogen for SpsD was mapped to residues 395-411 in the γ-chain, while a binding site for SpsD in Fn was localized to the N-terminal region. SpsD also binds to glycine-and serine-rich omega loops within the C-terminal tail region of cytokeratin 10 (16).

104 Another important Sps protein involved in the host colonization is SpsO, that has been
105 demonstrated to mediate adherence to *ex vivo* canine keratinocytes (12). However, the host
106 ligand(s) interacting with SpsO remain to be determined (15).

107 The SpsO protein of *S. pseudintermedius* is also likely to be involved in colonization of the
108 canine host. It promotes adhesion to *ex vivo* canine corneocytes, as does SpsD, although the
109 ligand(s) recognized by SpsO remain to be identified.Invasive bacteria actively induce their own
110 uptake by phagocytosis into normally non-phagocytic cells where they establish a protected niche
111 within which they can replicate (17). For example, *S. aureus*, usually considered an extracellular
112 pathogen, can invade a variety of non-professional phagocytic cells explaining its capacity to
113 colonize mucosa and its persistence in tissue after bacteraemia. The underlying major molecular
114 mechanism of invasion involves the Fn-binding adhesins FnBPA and FnBPB (18,19). Fn-bridging
115 between FnBPs and α5β1 integrins on the host cell surface is sufficient to induce zipper-type uptake
116 of staphylococci (18-20). The ternary complex promotes integrin clustering and a relay of signals
117 that result in cytoskeletal rearrangements. The rearrangements are accompanied by endocytosis of
118 *S. aureus* and internalization (17).

119 In this study we wished to investigate whether *S. pseudintermedius* shares with *S. aureus* the
120 ability to invade non-professional phagocytic cells and to determine the bacterial and host
121 components that are involved. We reasoned that both SpsD and SpsL could be involved in the
122 internalization of *S. pseudintermedius* by host cells. The objective of this study was to investigate
123 internalization and its mechanistic basis. The analysis of this process will provide insights into the
124 potential of a vaccine comprising components of SpsD and SpsL for the prevention of canine
125 pyoderma.

126

127 **MATERIALS AND METHODS**

128 **Bacterial strains and culture conditions.**

129 *S. pseudintermedius* strain ED99 (formerly M732 / 99) was isolated from a canine bacterial
130 pyoderma case presented to the Dermatology Service of The Hospital for Small Animals, Division
131 of Veterinary Clinical Sciences, The Royal (Dick) School of Veterinary Studies, The University of
132 Edinburgh. *S. pseudintermedius* strains 264, 324, 326, 327, 328 and 329 were isolated from cases of
133 canine pyoderma and were a kind gift from Dr. Neil McEwan, University of Liverpool. *S.*
134 *pseudintermedius* strains 81852, 91180, 253834, 237425, 235214/1 were isolated from cases of
135 canine pyoderna and were donated from Istituto Zooprofilattico Sperimentale della Lombardia e
136 della Emilia Romagna, Pavia, Italy. The strains were classified as *S. pseudintermedius* using
137 standard phenotypic tests (21). *S. pseudintermedius* ED99 and its mutants were grown in Brain
138 Heart Infusion (BHI) (VWR International Srl, Milan, Italy) at 37°C with shaking. Transformants of
139 *Lactococcus lactis* harbouring plasmid pOri23, pOri23::*spsD* or pOri23::*spsL* (15) were grown in
140 M17 medium (Difco, Detroit, MI, USA) supplemented with 10% lactose, 0.5% glucose and 10 µg
141 ml^{-1} erythromycin at 30°C without shaking. *Escherichia coli* DC10B (22) and TOPP3 (Stratagene,
142 La Jolla, CA) were grown in Luria agar (LA) and Luria broth (LB) (VWR International Srl).
143 **Reagents, proteins and antibodies.** Human fibronectin was purified from plasma by a
144 combination of gelatin- and arginine-Sepharose affinity chromatography. The purity of the protein
145 was assessed by 7.5% SDS-PAGE and Brilliant Blue Coomassie staining. To exclude the
146 possibility of trace amounts of contaminants, affinity purified fibronectin was spotted onto
147 nitrocellulose membranes at different concentrations and overlaid with anti-fibrinogen and anti-
148 plasminogen antibodies (23). The N-terminal fragment of Fn (N29) containing the five N-terminal
149 type I modules, and the gelatin-binding domain (GBD) consisting of four type I modules and two
150 type II modules were isolated as previously reported (24). Unless stated otherwise all reagents were
151 purchased from Sigma-Aldrich (St Louis, MO, USA). The anti-human Fn rabbit polyclonal IgG
152 was purchased from Pierce (Rockford, IL, USA). The mouse monoclonal antibody JBS5 against the
153 human integrin α5β1 was purchased from Merck-Millipore (Darmstadt, Germany). The rabbit
154 polyclonal antibody against the α5 chain of the α5β1 integrin, the mouse monoclonal antibodies

155 BV7 against the human β1 chain and B212 against the human β3 chain of the integrin αvβ3 were a
156 generous gift of Prof. G. Tarone (University of Turin, Italy). Mouse polyclonal antibodies against
157 region A of SpsD and SpsL were prepared as previously reported (16).
158 **DNA manipulation.** DNA encoding regions $SpsD_{164-523}$, $SpsD_{520-846}$, $SpsD_{844-960}$, $SpsL_{220-531}$, and
159 $SpsL_{538-823}$ were amplified by PCR using *S. pseudintermedius* ED99 genomic DNA as the template.
160 Oligonucleotides were purchased from Integrated DNA Technologies (Leuven, Belgium) (see
161 Table S1 in the supplemental material). Restriction enzyme cleavage sites (see Table S1) were
162 incorporated at the 5' ends of the primers to facilitate cloning into plasmid pQE30 (Qiagen,
163 Chatsworth, CA, USA). Restriction enzymes were purchased from New England Biolabs
164 (Hertfordshire, UK). The integrity of cloned DNA was confirmed by sequencing (Primmbiotech,
165 Milan, Italy).
166 **Expression and purification of recombinant proteins.** Recombinant proteins were expressed
167 from pQE30 in *E. coli* TOPP3 (Stratagene). Overnight starter cultures were diluted 1:50 in LB
168 containing ampicillin (100 μg ml^{-1}) and incubated with shaking until the culture reached OD_{600nm}
169 0.4-0.6. Recombinant protein expression was induced by addition of isopropyl 1-thio-β-D-
170 galactopyranoside (0.5 mM) and continued for 2 h. Bacterial cells were harvested by centrifugation
171 and frozen at -80°C. Recombinant proteins were purified from cell lysates by Ni^{2+} affinity
172 chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, UK). Protein
173 purity was assessed to be 98% by SDS-PAGE and Brilliant Blue Coomassie staining and
174 densitometric analysis.
175 **ELISA-type solid phase binding assays.** The ability of immobilized recombinant proteins to
176 interact with soluble human Fn was determined using ELISA assays. Microtiter wells were coated
177 overnight at 4°C with 100 μl of 10 μg ml^{-1} of bacterial protein in 50 mM sodium carbonate, pH 9.5.
178 To block additional protein-binding sites, the wells were treated for 1h at 22°C with 200 μl of 2%
179 BSA in PBS. The plates were then incubated for 1 h with increasing amounts of Fn. One microgram

of the specific anti-Fn rabbit IgG (1:2000) in PBS with 0.1% BSA was added to the wells, followed by incubation for 90 min. After washing, the plates were incubated for 1 h with peroxidase-conjugated secondary anti-rabbit IgG diluted 1:1000. After washing, *o*-phenylenediamine dihydrochloride was added and the absorbance at 490 nm was determined. To calculate the relative affinity association constant (K_A) values of each bacterial protein for Fn the following equation was employed: $A = A_{max}[L]K_A/(1 + K_A[L])$, where [L] is the molar concentration of ligand. The dissociation constants (K_D values) were calculated as reciprocals of the K_A values. The assays were performed at least 3 times for each protein.

Construction of *spsD* and *spsL* null mutants. Allele replacement mutagenesis of *spsD* and *spsL* was performed using the thermosensitive plasmids pIMAY and pIMAY-Z (Table 1). For generation of the *spsD* null mutation, approximately 500 bp fragments of DNA flanking the gene were PCR-amplified using the AB and CD primers, spliced together and cloned into the blunt end ligation pSC-B vector (StrataClone, Agilent Technologies, Santa Clara, CA) before subcloning into pIMAY to produce the pIMAY Δ*spsD* construct. The plasmid was transformed into *E. coli* DC10B before being electro-transformed into *S. pseudintermedius* ED99 at 28°C selecting on 10 µg ml^{-1} chloramphenicol, as previously described for *S. aureus* (25). For generation of the *spsL* null mutation, a sequence ligase independent cloning (SLIC) protocol was performed (26) using pIMAY-Z, a derivative of pIMAY with a constitutive *lacZ* marker, to construct pIMAY-Z Δ*spsL*. Once the plasmids were transformed into ED99 at 28°C, growth at the restrictive temperature of 37°C selected for integrants. OUT primers, located outside of the flanking regions and gene of interest, were used to determine if integrationhad occurred upstream or downstream of the chromosomal gene (22). A single colony from each site of integration was inoculated into broth and grown at 28°C then diluted and grown at 37°C. The *S. aureus* antisense *secY* mechanism within pIMAY (27) was non-functional in *S. pseudintermedius* and the *lacZ* marker was ineffective

because plasmid-free cells expressed endogenous β-galactosidase activity. Allele exchange was confirmed using OUT primer PCR and sequencing the resultant fragment (see Table S2).

Release of surface proteins from *S. pseudintermedius* and *L. lactis*. *S. pseudintermedius* and *L. lactis* cells were grown to OD_{600nm} of 0.4-0.6. Cells were harvested by centrifugation at 7000 x *g* at 4°C for 15 min, washed 3 times with PBS and resuspended at an OD_{600nm} of 40 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with 30% raffinose. Cell wall proteins were solubilized from *S. pseudintermedius* by incubation with lysostaphin (200 µg ml^{-1}) and from *L. lactis* with mutanolysin (1000 U/ml) and lysozyme (900 µg ml^{-1}) at 37°C for 20 min in the presence of protease inhibitors (Complete Mini TM; Roche Molecular Biochemicals, Indianapolis, IN, USA). Protoplasts were recovered by centrifugation at 6000 x *g* for 20 min, and the supernatants were taken as the wall fractions. The material obtained from *S. pseudintermedius* ED99 and its mutants were adsorbed on IgG-sepharose columns before Western immunoblotting analysis to remove IgG-binding proteins that would otherwise interfer with the specific antibody staining.

SDS-PAGE and Western immunoblotting. Samples for analysis by SDS-PAGE were boiled for 5 min in sample buffer [0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue] and separated on 10% (w/v) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue (BioRad, Hercules, CA, USA). For Western immunoblotting, material was subjected to SDS-PAGE and then electroblotted onto a nitrocellulose membrane (GE Healthcare). The membrane was blocked overnight at 4°C with 5% (w/v) skim milk in PBS, washed, and incubated with mouse polyclonal antibody against region A of SpsD or SpsL (1 µg ml^{-1}) for 1 h at 22°C. Following additional washings with 0.5% (v/v) Tween 20 in PBS (PBST), the membrane was incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG. Finally, blots were developed using the ECL Advance Western Blotting Detection Kit (GE Healthcare) and an ImageQuantTM LAS 4000 mini Biomolecular Imager (GE Healthcare).

Mammalian cell lines and culture conditions. Canine progenitor epidermal keratinocytes (CPEK) cells were cultured in *CnT-0.9* medium (CELLnTEC, Bern, Switzerland), without antibiotics at 37°C in 5% CO_2. The spontaneously immortalised keratinocyte (HaCaT) and the human epithelial cell line HEp-2 were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Milan, Italy), 2% penicillin and streptomycin, 2% sodium pyruvate, 2% L-glutamine at 37°C in 5% CO_2. Cells were cultured in T75 flasks to approximately 95% confluency, liberated with trypsin-EDTA (EuroClone), resuspended in invasion medium (growth medium without antibiotics) and plated as reported below in the cell invasion assay.

Cell invasion assay. Cell invasion assays were performed (28) with modifications. Briefly, cells were plated at 5 x 10^5 (in 0.4 ml invasion medium) into 24 well plates (Corning) and allowed to attach for 24 h at 37°C. Staphylococcal cultures were grown overnight in BHI at 37°C with shaking. *L. lactis* was grown overnight in M17 broth at 30°C without shaking. The following day, cultures were diluted 1:40 in fresh BHI or M17 medium, respectively, and grown to an OD_{600nm} of 0.4, were washed 3 times in PBS and diluted to obtain 10^7 cells/ml in *CnT-BM.2* supplemented with 10% FBS + 2 mM L-glutamine. Bacterial suspensions (1 ml) were added to each well and the plates incubated for 2 h at 37°C. Monolayers were then washed 3 times in PBS to remove unattached bacteria. Media containing antibiotics (200 µg ml^{-1} gentamicin + 2% penicillin and streptomycin) was added and the plate incubated for an additional 2 h to kill extracellular bacteria. The wells were washed again, and internalized bacteria released by incubating with 200 µl of H_2O containing 0.1% v/v Triton-X 100. Serial dilutions of the cell lysates were plated in duplicate on BHI agar and CFU were counted after incubation. All assays were carried out in triplicate. Samples of monolayers were lysed prior to inoculation, plated on BHI agar and the absence of staphylococcal colonies noted.

Inhibition of invasion. The Src family kinase inhibitors PP2, PP3 and CGP77675 (25 µM) were dissolved in dimethyl sulphoxide (DMSO), added to the cell media at the indicated concentrations

and preincubated with monolayers for 1 h at 37°C in 5% CO_2 before addition of bacteria. Likewise, wortmannin (20 nM), genistein (200 µM) and cytochalasin D (50 µM) were dissolved in PBS and incubated with cells for 60 min prior to the addition of bacteria. Gentamicin protection assays were then performed as described above except no intermediate washing was carried out. To test cell viability during exposure to the Src inhibitors the compounds were added to cell monolayers for 3 h at 37°C. Then the cells were gently washed with DMEM, trypsinized and mixed with an equal volume of trypan blue (0.5% (v/v) in PBS) for 5 min. Ten microliters of the mixture were placed on a Neubauer chamber and stained cells were counted by light microscopy. The percentage of dead cells was calculated by dividing the mean number of dead (stained) cells by the total number of cells in 50 microscopic fields, and multiplying by 100.

Fluorescence microscopy. Bacteria were grown to an OD_{600nm} of 0.3 (*S. pseudintermedius*) or 0.4 (*L. lactis* centrifuged resuspended in 100 µl PBS. Then 0.5 µl 10 mM calcein-AM (Molecular Probes, Eugene, OR, USA) was added and incubated for 1 h at 37°C (*S. pseudintermedius*) or 2 h at 30°C (*L. lactis*). Stained bacteria were washed 3 times with PBS and resuspended in 1 ml PBS. Suspensions (100 µl) were added to CPEK monolayers and incubated for 2 h at 37°C to allow internalization. Cells were washed with PBS, counterstained for 1–3 min with ethidium bromide (10 µg ml^{-1}) and washed again. Fluorescence microscopy (Olympus BX51; Olympus, Segrate, Italy) was performed using a green filter, a red filter, and white light. Images were captured with a CCD camera and assembled using Adobe Photoshop Creative Suite 2.

Staining of monolayers. Mammalian cells were stained with Giemsa Stain Modified Solution (Sigma) according to the manufacturer's instructions and observed under a light microscope at 20 x magnification.

Invasion assays with formaldehyde-fixed staphylococci. To perform invasion assays with killed bacteria, staphylococci were fixed in 0.5% formaldehyde in PBS for 1 h, stained with Calcein-AM and subjected to fluorescence microscopy. Alternatively, to analyze the effect of formaldehyde-

fixed staphylococci on cell survival, monolayers were stained with Giemsa and observed as reported above.

MTT assay. The MTT tetrazolium reduction assay was performed according to the manufacturer's instructions (Sigma).

Statistical methods. Continuous data were expressed as means and standard deviations. Two-group comparisons were performed by Student's *t* test. One-way analysis of variance, followed by Bonferroni's post hoc tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed *P* values of 0.001 were considered statistically significant.

RESULTS

SpsD and SpsL binding to fibronectin. To localize the Fn-binding sites in SpsD and SpsL, recombinant domains were obtained following PCR amplification of genomic DNA from strain ED99. The cloned SpsD domains included the minimun fibrinogen-binding region (residues 164-523) ($SpsD_{164-523}$), a connecting region C (residues 520-846) ($SpsD_{520-846}$) and a repeat region R (residues 844-960) ($SpsD_{844-960}$). Two recombinant SpsL domains were expressed: the N-terminal region encompassing residues 220-531 ($SpsL_{220-531}$) and the repetitive domain spanning 538-823 ($SpsL_{538-823}$). As shown in ELISA–type solid phase binding assays (Fig. 2) recombinant $SpsD_{520-846}$ and $SpsL_{538-823}$ regions bound Fn dose-dependently and saturably, while no binding was exhibited by $SpsL_{220-531}$ and $SpsD_{844-960}$. $SpsD_{520-846}$ and $SpsL_{538-823}$ domains bound Fn in the low nanomolar range ($SpsD_{520-846}$ K_D = 1.7±0.38, $SpsL_{538-823}$ K_D = 0.81±0.02 nM) while $SpsD_{164-523}$ gave a half maximal binding of 2.19±0.47 µM (data not shown).

Invasion of mammalian cells by *S. pseudintermedius*.

Several *S. pseudintermedius* isolates were found to invade canine keratinocyte-derived CPEK cells. The magnitude of invasion was very similar to or even higher than that of the archetypal invasive *S.*

aureus strain Cowan 1 (Fig. 3). Thus, invasion of CPEK cells is a general property of *S. pseudintermedius*.

Requirement for fibronectin for efficient invasion by *S. pseudintermedius* ED99. To investigate the role of soluble plasma-derived Fn in invasion, FBS was passed over a gelatin-Sepharose column to remove soluble Fn before being used in the invasion assay. An 85 % reduction in the level of invasion of CPEK cells was observed when the Fn-depleted FBS was used in the invasion medium compared to unadsorbed FBS. The addition of human Fn to a final concentration of 1µg ml^{-1} was sufficient to restore the level of invasion to that observed in the presence of whole FBS (Fig. 4A). This suggests that *S. pseudintermedius* strain ED99 can use soluble plasma-derived Fn in the invasion process. Removal of FBS from the assay reduced the invasion level by 95%, suggesting that additional minor components in the FBS other than Fn might contribute. However, although removal of Fn from the gelatin-adsorbed FBS was shown by ELISA and Western blotting, residual internalization can be due to trace amounts of Fn remaining in the invasion medium.

When bacteria were preincubated with increasing amounts of the N29 fragment of Fn and then tested for adherence to or invasion of CPEK cells, we observed an almost complete inhibition of bacterial internalization. Conversely, no effect was observed when the invasion assay was performed with a high concentration of the GBD of Fn (Fig. 4B). Together these findings indicate that the N29 domain is specifically involved in adhesion to and invasion of CPEK cells.

Invasion of CPEK cells by ED99 mutants and *L. lactis* expressing SpsD or SpsL. Mutants of *S. pseudintermedius* ED99 deficient in SpsD and SpsL were tested for their ability to attach to surface-coated Fn. Mutants defective in either SpsD or SpsL alone adhered equally as well as the parental strain while the double mutant defective in both proteins did not bind at all (data not shown). The absence of SpsD or SpsL proteins was confirmed by testing material solubilized from the cell wall with lysostaphin by Western blotting and probing with antibodies against region A of SpsD or SpsL. Both the proteins were absent from the double mutant (Fig. 5A). Conversely, SpsL was expressed normally by the SpsD mutant and *vice versa*.

The SpsD and SpsL defective mutants were also tested for invasiveness. Single mutants retained the ability to invade CPEK cells at the same level as the wild type, while the double mutant lacking both SpsD and SpsL invaded at a much lower level (Fig. 5B).

In order to test whether co-receptors are required for SpsD or SpsL-mediated invasion, we expressed both *S. pseudintermedius* proteins individually in *Lactococcus lactis* (Fig 6A). Transformants of *L. lactis* carrying plasmid pOri23::*spsD* and pOri23::*spsL* (Fig. 6A) showed invasiveness similar to that of the wild type strain ED99 (Fig. 5B), while very low internalization by CPEK cells was observed with *L. lactis* harboring the empty plasmid (Fig. 6B).

Reduced invasion by the double mutant of ED99 was also assessed by visualizing uptake into CPEK cells by fluorescent imaging. Bacteria were stained with calcein-AM (green) prior to CPEK cell invasion and at the assay end-point the fluorescence of external bacteria was visualized with ethidium bromide (red). As shown in Fig. 5 C, the wild type and the single mutant strains were observed inside CPEK cells, while no green fluorescence, indicative of the internalized bacteria, was detected when the double mutant was tested. *L. lactis* expressing SpsL or SpsD behaved similarly (Fig. 6C). Together these results demonstrate that expression of a single adhesin (SpsD or SpsL) is sufficient to confer efficient uptake of bacteria into CPEK cells.

Localization of Sps domains promoting invasion of CPEK cells. To identify the domains of SpsD and SpsL that are involved in invasion, recombinant fragments were assessed for inhibition of *S. pseudintermedius* ED99 uptake into CPEK cells. We found that $SpsD_{520-846}$ (Fig.7A) and $SpsL_{538-823}$ (Fig. 7B) strongly inhibited internalization, whereas $SpsD_{164-523}$ showed a weak inhibitory effect. The inhibitory effects exhibited by these proteins correlates with their affinities for fibronectin. $SpsL_{220-631}$ and $SpsD_{844-960}$ when used at the same concentrations did not interfere with staphylococcal invasion.

Dependence of invasion on integrin $\alpha_5\beta_1$. Immunofluorescent antibodies that specifically bind to the α_5 subunit of the human Fn-binding $\alpha_5\beta_1$ integrin stained CPEK cells, suggesting that the canine cells express an $\alpha_5\beta_1$ integrin that is closely related to the human integrin (Fig. 8A, inset). To

test the role of the $\alpha_5\beta_1$ integrin in invasion, CPEK cells were preincubed with function-blocking anti–$\alpha_5\beta_1$ IgG prior to adding *S. pseudintermedius*. Antibodies recognizing the α_5 and the β_1 chains both reduced internalization of *S. pseudintermedius* by more than 80%, whereas antibodies against the β_3 chain of the human $\alpha_v\beta_3$ integrin did not alter invasion (Fig. 8A). This indicates that the $\alpha_5\beta_1$ integrin on canine CPEK cells is responsible for Fn-mediated bacterial invasion.

Inhibition of invasion by an RGD-containing peptide. The $\alpha_5\beta_1$ integrin recognizes the tripeptide sequence RGD within the cell-binding domain of Fn (29, 30). To investigate the role of this interaction in invasion of *S. pseudintermedius*, the effect of the RGDS peptide was analyzed. Incubation of CPEK cells with the RGDS peptide reduced the level of invasion by strain ED99 in a dose-dependent manner, while a control peptide RGES had no inhibitory effect (Fig. 8B). This suggests that the interaction of $\alpha_5\beta_1$ with Fn is necessary for efficient invasion of CPEK cells.

Protein phosphorylation during *S. pseudintermedius* invasion. To identify changes in host cell signalling associated with staphylococcal invasion, the assay was performed in the presence of protein tyrosine phosphorylation inhibitors. Genistein, a tyrosine kinase inhibitor, strongly inhibited internalization, whereas wortmannin, an inhibitor of the phosphatidylinositol-3-phosphate kinase, did not (Fig. 8C). We also tested Src kinase inhibitors and found that both CGP77675 and PP-2 inhibited *S. pseudintermedius* internalization into CPEK cells. While both inhibitors effectively blocked internalization, CGP77675 appeared to be a more potent inhibitor compared to PP-2. PP-3, a compound similar to PP-2 but with no significant Src inhibitory activity, had no effect on internalization (Fig. 8C). At the concentrations used the inhibitors did not affect bacterial adhesion to the CPEK cells or cause loss of viability as shown by Trypan blue staining (data not shown). To investigate a possible role for actin cytoskeleton rearrangements in *S. pseudintermedius* invasion, we tested cytochalasin D which interferes with F-actin polymerization. Pre-treatment of CPEK cells with 1µg/ml cytochalasin D almost completely abolished invasion demonstrating the involvement of actin cytoskeletal rearrangements (Fig. 8C).

Invasion of Hep-2 and HaCaT cell lines by *S. pseudintermedius*. Human-derived Hep-2 and HaCaT cells were efficiently invaded by *S. pseudintermedius*, and invasion was dependent on Sps proteins. In addition, as reported for CPEK cells, internalization required the presence of Fn and involved the $\alpha_5\beta_1$ integrin (see Figs. S1 and S2).

Alterations to cell monolayers following internalization by *S. pseudintermedius*. To investigate alterations to CPEK, Hep-2 and HaCaT cells following *S. pseudintermedius* invasion, cell monolayers were infected with *S. pseudintermedius* strain ED99 for 2 h prior to gentamicin treatment. Then the cells were incubated for 4 h and 36 h, fixed and analyzed for morphological changes by light microscopy. A remarkable difference in morphology was observed between infected and uninfected cell monolayers. Internalization of bacteria by CPEK cells caused cell detachment and a reduction of the cell density (Fig. 9A). Incubation of Hep-2 cells with strain ED99 for 36 h resulted in rounding and detachment of the cells (see Fig. S3, panel A). In contrast, HaCaT cells showed a pattern similar to that exhibited by uninfected cells (see Fig. S3, panel C).

The assessment of the cell growth and survival of infected cells by the MTT assay showed that staphylococcal invasion substantially reduced the viability of CPEK (Fig. 9B) and Hep-2 (see Fig. S3, panel B) cells, whereas HaCaT cells survived to a level comparable to that of the uninfected cells (see Fig. S3, panel D). To further investigate the contribution of bacterial invasion to cell damage, all the cell lines were incubated with the *spsDspsL* double mutant. Both morphological observations and MTT assays showed that the double mutant did not affect the viability of cells.

DISCUSSION

In this paper we have analysed the molecular mechanism by which *S. pseudintermedius* adheres to and invades canine keratinocytes (CPEK cells) and the effects of internalization on the viability of the mammalian cells. We found that all strains of *S. pseudintermedius* tested invaded CPEK cells efficiently. Importantly, we found that both the cell wall-anchored surface proteins SpsD and SpsL

efficiently promoted invasion of strain ED99. Single mutants defective in either SpsD or SpsL alone showed no reduction in invasion. Only the double mutant lacking both proteins was defective. Conversely, both SpsD and SpsL promoted efficient uptake of the non-invasive surrogate host *L. lactis* when expressed ectopically from recombinant plasmids. Subdomains within SpsD and SpsL were expressed as recombinant proteins which allowed identification of regions with a high affinity for Fn and which also strongly inhibited bacterial invasion. Invasion of CPEK cells was dependent on the presence of Fn as demonstrated by the markedly reduced uptake upon removal of Fn from the cell culture medium (fetal bovine serum) and the restoration of invasion by supplementation with purified human Fn. We recognize the limitation of using human and bovine Fn to assess the role of this protein in bacterial invasion of a canine cell line. However, it should be noted that there is a high level of similarity between human, bovine and canine Fn (93-94% identity, 98% similarity) so that the use of human or bovine Fn is valid.

In *S. aureus* Fn-binding proteins FnBPA and FnBPB both promote invasion into mammalian cells where Fn acts as a bridge between the bacterial surface protein which binds to the N terminal N29 domain by the tandem β zipper mechanism and the α5β1 integrin which recognizes an RGD motif within the C-terminal repeat 10 of Fn (18, 33). The finding that the N-terminal region of Fn and an RGD containing peptide inhibited *S. pseudintermedius* invasion into CPEK cells strongly suggests that the same mechanism is employed involving the Fn binding domains of SpsD and SpsL. Inhibition of invasion of CPEK cells by monoclonal antibodies recognizing epitopes in the human α5β1 integrin strongly suggests that the canine CPEK cells express an immunocrossreactive integrin that is responsible for bacterial adhesion and invasion.

The Fn bridging mechanism for attachment to and invasion of mammalian cells results from integrin-initiated actin polymerization stimulated by receptor clustering and cell signalling events involving Src (34, 35). Here invasion of *S. pseudintermedius* was strongly reduced by the Src-specific inhibitors CGP77675 and PP-2 implying that similar mechanisms are responsible. Similar results were obtained when human Hep-2 epithelial cells and HaCaT keratinocytes were tested for

433 invasion by *S. pseudintermedius* ED99. However, certain differences were observed when
434 compared to invasion of CPEK cells. A ten-fold smaller inoculum was needed for efficient invasion
435 of Hep-2 cells compared to HaCaT and CPEK cells. We speculate that the differences in invasion
436 efficiencies might be due to variations in the density of the α5β1 integrins although other factors
437 could be involved. Together this data demonstrates that *S. pseudintermedius* employs a similar
438 mechanism of host cell invasion as *S. aureus* that involves bacterial surface proteins binding to Fn
439 and uptake mediated by integrin α5β1.
440 Invasion of CPEK and Hep-2 cells resulted in cells detaching and loosing viability whereas HaCaT
441 cells remain unchanged. Thus in the first two cells lines invasion by *S. pseudintermedius* triggers a
442 reduction in cell viability. Formaldehyde-killed bacterial cells were actively internalized by the
443 mammalian cells suggesting that no active expression of invasogenic factors was necessary to
444 achieve invasion. Conversely, the lack of effects on host cell survival by killed bacterial cells
445 indicates that additional factors such as secreted toxins are required to induce cell death.
446 Membrane damaging toxins that are expressed by intracellular *S. aureus* are major factors in
447 promoting apoptosis (36). *S. pseudintermedius* has the potential to express a bicomponent
448 leukotoxin Luk-I which is similar to the Panton Valentine leucocidin (PVL) of *S. aureus* (37) as
449 well as a homologue of β-toxin and a putative haemolysin (haemolysin III) (8). It can be
450 hypothesized that at least one of these factors is responsible for inducing cell death in CPEK cells.
451 Indeed, PVL facilitates escape of *S. aureus* from human keratinocyte endosomes and induces
452 apoptosis (36) which might indicate a role here for Luk-I. Studies with mutants lacking one or more
453 of the toxins will help clarify this point.
454 The initiation of the skin infection canine pyoderma is probably related to the ability of *S.*
455 *pseudintermedius* to adhere to corneocytes on the surface of the *stratum corneum* as well as to
456 invade the underlying keratinocytes. *S. pseudintermedius* adheres more strongly to corneocytes
457 from regions of inflamed skin of dogs with atopic dermatitis than to non-inflamed areas suggesting

that ligands for bacterial surface protein adhesins are present at higher levels (31). Both SpsD and SpsO mediate bacterial adherence to canine corneocytes but the host ligands involved are not known (12). In addition, fibronectin is present in the *stratum corneum* of atopic human skin where it could provide an abundant ligand whereas it was not detected in healthy skin (32). Thus Fn could promote colonization of the *stratum corneum* as well as invasion of keratinocytes.

In conclusion, we have identified and characterized two fibronectin-binding proteins of *S. pseudintermedius* which are required for adhesion to and invasion of keratinocytes. An appropriate animal model will be required to assess the significance of SpsD and SpsL in the pathogenesis of canine pyoderma and to establish whether these antigens are suitable candidates for a multicomponent vaccine to combat the disease.

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585

586 **FIGURE LEGENDS**

587 **FIG 1** Schematic diagram of SpsD and SpsL proteins from *S. pseudintermedius* ED99. The A
588 domain of SpsD spans residues 37-519 following the secretory signal sequence S followed by a
589 connecting domain C (residues 520-866) and a repeat region R. A sorting signal (SS:LPXTG motif,
590 hydrophobic domain and positively charged residues) occurs at the extreme C-terminus. SpsL
591 includes a signal sequence S at the N terminus followed by an A domain (residues 39-531), a
592 domain containing seven tandem repeats (domain R, residues 543-818) and a C-terminal sorting
593 signal (SS). The A domains of both proteins align with A domains of the MSCRAMM family of *S.*
594 *aureus* surface proteins and each comprise three subdomains N1, N2 and N3 with N2 and N3
595 predicted to form IgG-like folds. The A domains of SpsD and SpsL from ED99 have 30% identity
596 and 50% similarity. The recombinant proteins are indicated along with ability of each truncate to
597 bind to fibronectin.
598

599 **FIG 2** Dose-dependent binding of fibronectin to SpsD and SpsL fragments in an ELISA-type assay**.**
600 Microtiter wells were coated with $SpsD_{520-846}$, $SpsD_{844-960}$, $SpsL_{220-531}$, and $SpsL_{538-823}$. The wells
601 were probed with increasing amounts of Fn, followed by incubation with rabbit anti-Fn IgG and
602 HRP-conjugated goat anti-rabbit IgG. The graph is representative of three experiments with each
603 point representing the average of triplicate wells.
604

605 **FIG 3** Invasion of CPEK cell monolayers by *S. pseudintermedius* strains**.** *S. pseudintermedius* cells
606 were incubated with CPEK cell monolayers. Extracellular bacteria were killed with gentamicin and
607 internalized bacteria were quantified by plating lysates on BHI agar. The assay was performed

three times. Each point represents the average value for three replicas and error bars show the standard deviation. Statistically significant (*P*-value < 0.05, Student's *t*-test) differences in values compared with the control value (*S. aureus* Cowan 1) are indicated by an asterisk. The absence of intracellular bacteria in cell cultures was established by lysing samples of the confluent monolayers and plating on BHI agar prior to staphylococcal inoculation.

FIG. 4. Role of soluble fibronectin on invasion by *S. pseudintermedius*. A) To evaluate the effect of endogenous fibronectin, the invasion assay was performed in the presence of 10% FBS (a), 10 % Fn-depleted FBS (b), 10 % Fn-depleted FBS + 1µg/ml soluble human plasma Fn (c), and in the absence of FBS (d). Bacteria were incubated with CPEK cell monolayers and internalized bacteria were quantified as described in Fig. 3. Invasion is expressed as a percentage of that observed in the presence of 10% whole FBS (control: $2x10^5$ CFU). Each point represents the average value for three replicas and error bars represent means ± S.D. of three independent experiments performed in triplicate. Statistically significant (*P*-value < 0.01, Student's *t*-test) differences in values compared with control value in the presence of FBS is indicated by an asterisk. B) To determine the effect of the N29 fragment, staphylococci were incubated with increasing concentrations of N29 or 650 nM GBD for 30 min at 22°C. Bacteria were then added to CPEK cell monolayers and incubated at 37°C for 2 h and internalized bacteria were quantified as described in Fig. 3. Invasion is expressed as a percentage of that observed in the absence of inhibitors (control: $2.5x10^5$ CFU). Error bars represent means ± S.D. of three independent experiments performed in triplicate.

FIG 5 Invasion of CPEK cell monolayers by *S. pseudintermedius* and its mutants. A) Expression of SpsD and SpsL proteins. Cell wall proteins from the the wild type and mutants were solubilized with lysostaphin, separated by SDS-PAGE and analyzed by Western immunoblotting using mouse anti-SpsD or anti-SpsL and HRP-labeled rabbit anti-mouse IgG. B) *S. pseudintermedius* wild-type and Δ*spsD* Δ*spsL* were incubated with CPEK cell monolayers, and internalized bacteria quantified

as reported in Fig. 3. Error bars are ± S.D. of the mean of three independent determinations performed in triplicate. An asterisk indicates a significant difference (*P*-value < 0.05, Student's *t*-test) compared with the control (invasion with the wild type strain). C) Fluorescent microscopy investigating the contribution of SpsD or SpsL to interactions with CPEK cells. Confluent CPEK monolayers were incubated with calcein-AM labeled *S. pseudintermedius* to allow internalization, washed with PBS and counterstained with ethidium bromide. Green fluorescence represents intracellular staphylococci and red fluorescence extracellular bacteria. Scale bar = 40 µm.

FIG 6 Invasion of CPEK cell monolayers by *L. lactis.* A) Expression of SpsD and SpsL by *L. lactis*. Cell wall proteins were solubilized with mutanolysin and lysostaphin, separated by SDS-PAGE and analyzed by Western immunoblotting using mouse anti-SpsD or anti-SpsL as primary antibodies and HRP-labeled rabbit anti-mouse IgG. B) CPEK invasion by *L.lactis* expressing SpsD or SpsL. *L. lactis* were incubated with CPEK cell monolayers and internalized bacteria measured as indicated above. Error bars are ± S.D. of the mean of three independent determinations performed in triplicate. An asterisk indicates a significant difference (*P*-value < 0.05, Student's *t*-test) compared with *L. lactis* pOri23::*spsD* or pOri23::*spsL*. C) Fluorescent imaging investigating the contribution of SpsD or SpsL to interactions with CPEK cell monolayers. Confluent CPEK monolayers were incubated with calcein-AM-labeled *L. lactis* to allow internalization. Fluorescence was visualized as in Fig. 5. Scale bar = 40 µm.

Fig 7 Effect of SpsD and SpsL fragments on invasion of CPEK cells by *S. pseudintermedius.* CPEK cell monolayers were incubated with increasing concentrations of $SpsD_{164-523}$, $SpsD_{520-846}$ or $SpsD_{844-960}$ (A), $SpsL_{220-531}$ or $SpsL_{538-823}$ (B) prior to addition of bacteria. Invasion is expressed as a percentage of that observed in the absence of potential inhibitors (control: $2.2x10^5$ CFU). Error bars are ± S.D. of the mean of three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t*-test, **P* < 0.05, ***P* < 0.001).

660

661 **FIG 8** A) Effect of anti-integrin antibodies on invasion of CPEK cells by *S. pseudintermedius.*
662 CPEK monolayers were incubated with antibodies against $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins prior to the
663 addition of bacteria. After incubation internalized bacteria were quantified as described above.
664 Invasion is expressed as the percentage of that observed in the absence of antibodies (control:
665 $2.8x10^5$ CFU). Error bars represent the mean ± S.D. of three independent determinations performed
666 in triplicate. Statistically significant differences are indicated (Student's two-tailed *t*-test, $*P <$
667 0.05). The inset shows expression of $\alpha_5\beta_1$ integrin by CPEK cells by staining with
668 immunofluorescent antibodies that specifically bind to the α_5 subunit of the $\alpha_5\beta_1$ integrin. Scale bar
669 = 40 µm. B). Effect of an RGD containing peptide on invasion of CPEK cells by *S.*
670 *pseudintermedius* - CPEK cells were incubated with increasing concentrations of the RGDS or
671 RGES peptides prior to addition of bacteria. After incubation internalized bacteria were quantified
672 as described above. Invasion is expressed as a percentage of that observed in the absence of
673 peptides (control: $2.3x10^5$ CFU). Error bars represent the mean ± S.D. of three independent
674 determinations performed in triplicate. C) Effect of kinase inhibitors on invasion of CPEK cells by
675 *S. pseudintermedius* - CPEK cells were exposed to genistein, CGP77675, PP2 and PP3, wortmannin
676 and cytochalasin D for 1 h before addition of bacteria. Invasion assays were performed on inhibitor-
677 treated cells three times with similar results. Invasion is expressed as a percentage of that observed
678 in the absence of inhibitors (control: $2.1x10^5$ CFU). Error bars represent the mean ± S.D. of three
679 independent determinations performed in triplicate. Statistically significant differences are indicated
680 (Student's two-tailed *t*-test, $*P < 0.05$, $**P < 0.001$).

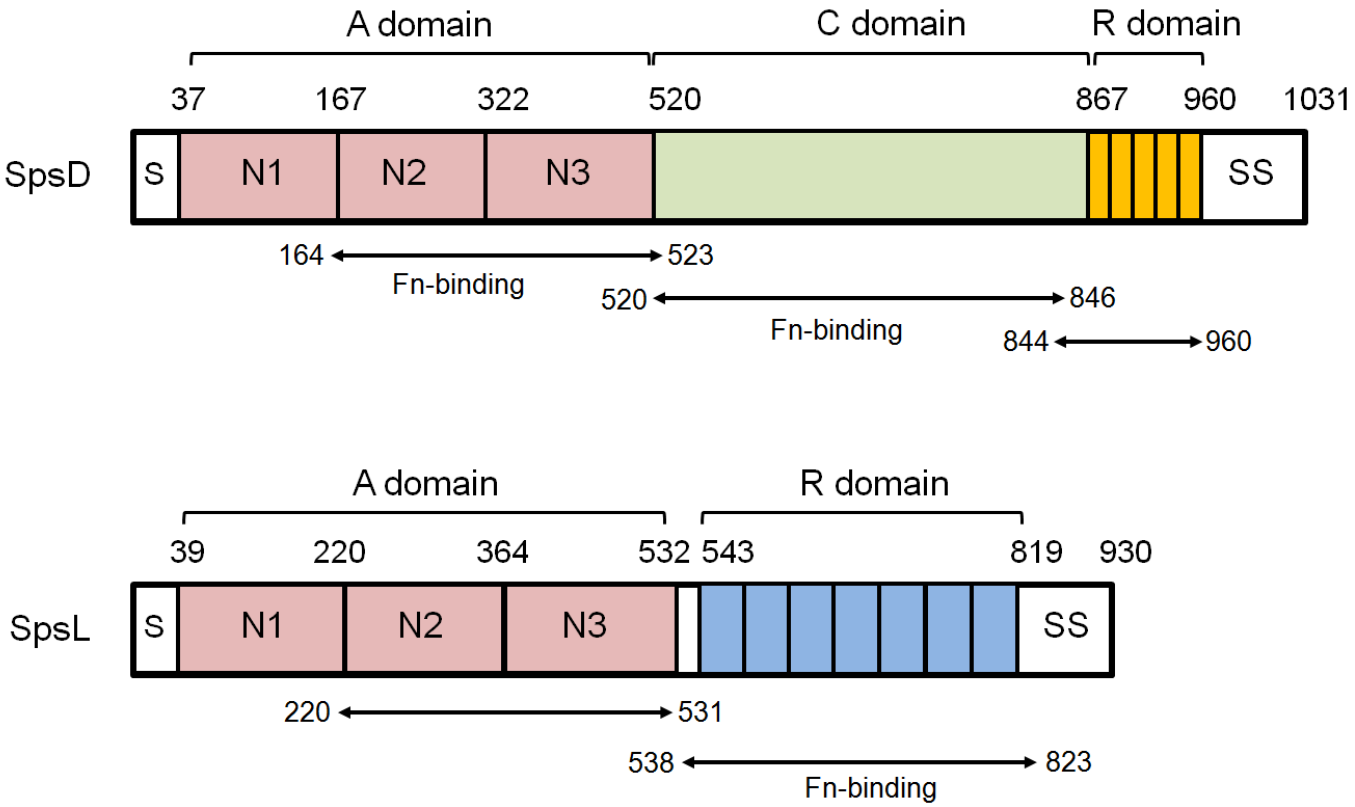
681

682 **FIG 9** Effects on CPEK cells following invasion. A) Alterations to CPEK monolayers after
683 infection with *S. pseudintermedius* and the double *ΔspsD/spsL* mutant. Monolayers were infected
684 with *S. pseudintermedius* prior to gentamicin treatment. Then the cells were incubated for 4 h and

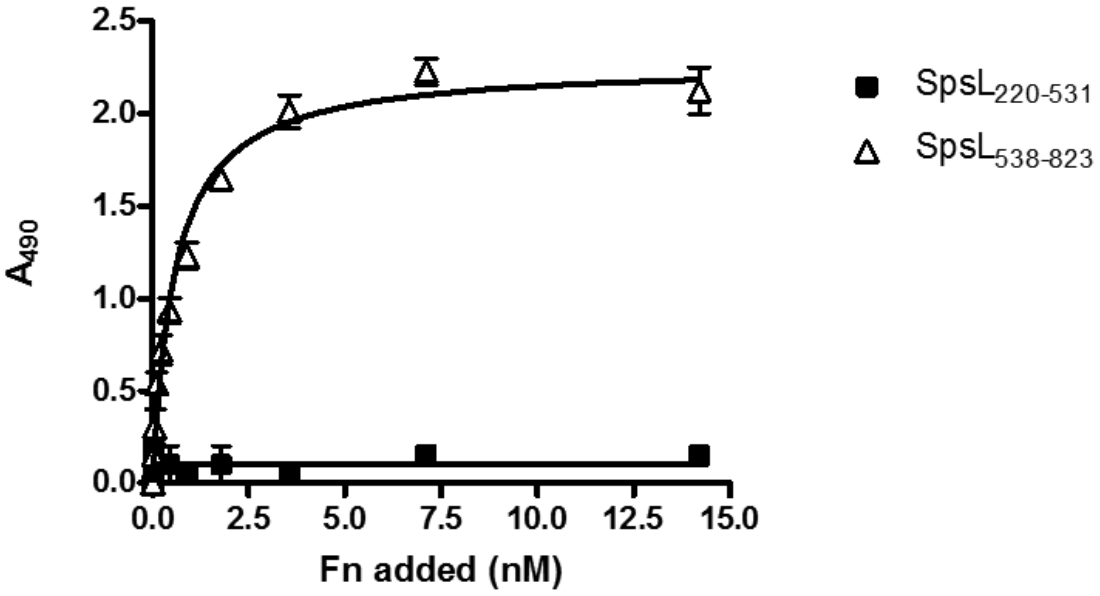
36 h, stained with Giemsa and observed for morphological changes by light microscopy. Scale bar = 200 µm. B) Assessment of survival of infected cells. Confluent CPEK cells were infected for 2h with *S. pseudintermedius* ED99 or the double *ΔspsD/spsL* mutant and then examined at 4h and 36 h after infection for viability by the MTT assay. Viability was assessed as the percentage of absorbance at 575 nm of treated cells relative to that of solvent-treated controls. Error bars represent the mean ± S.D. of three independent determinations performed in triplicate. Statistically significant differences are indicated (Student's two-tailed *t*-test, $*P < 0.05$).

TABLE 1 Plasmids used for the construction of *spsD* and *spsL* null mutants

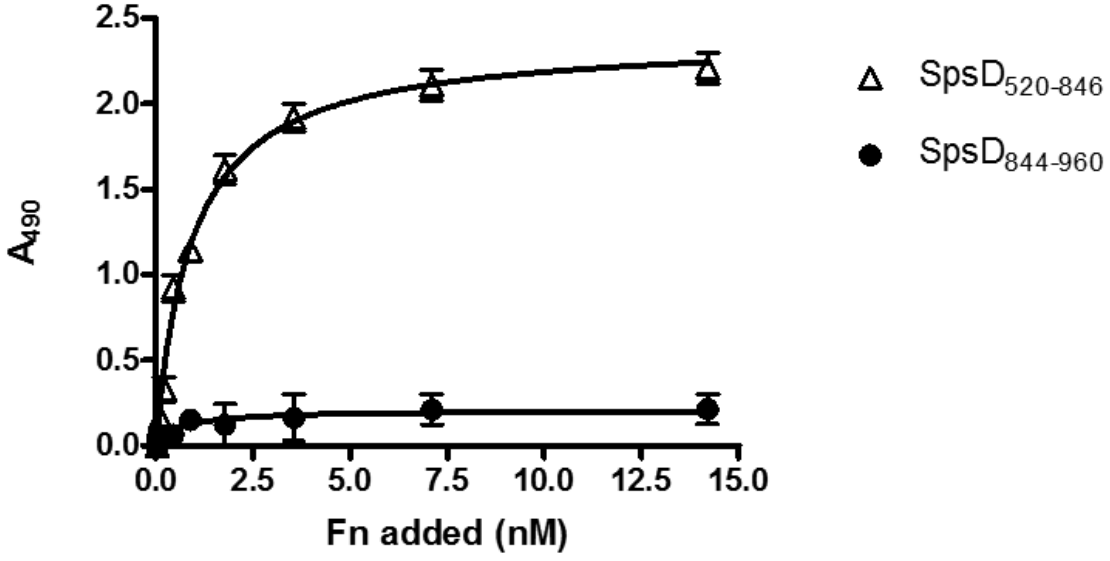
Strain or Plasmid	Description	Reference
pIMAY	Thermosensitive plasmid for allelic exchange	(21)
pIMAY Δ*spsD*	pIMAY with fragments flanking *spsD*	This paper
pIMAY-Z	pIMAY derivative with a constitutive *lacZ* marker	(25)
pIMAY-Z Δ*spsL*	pIMAY-Z with fragments flanking *spsL*	This paper

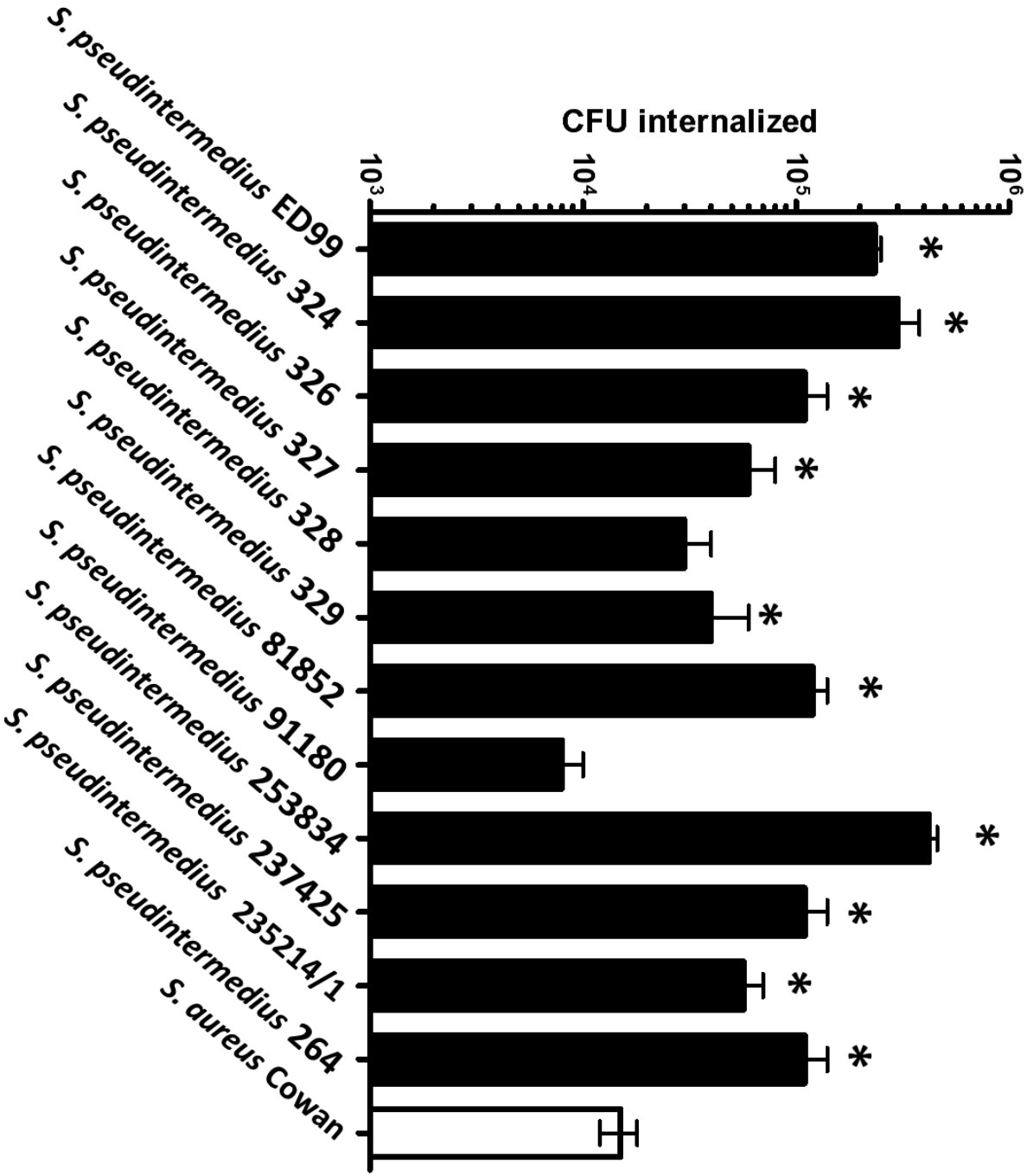


A



B





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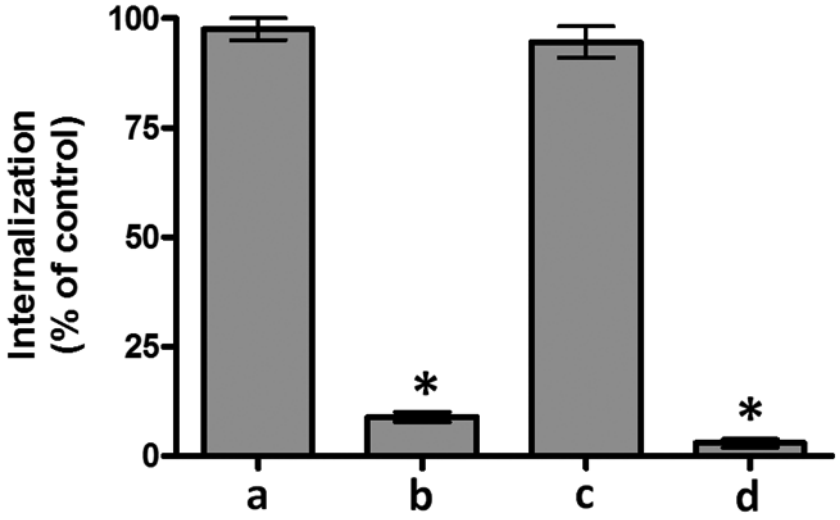

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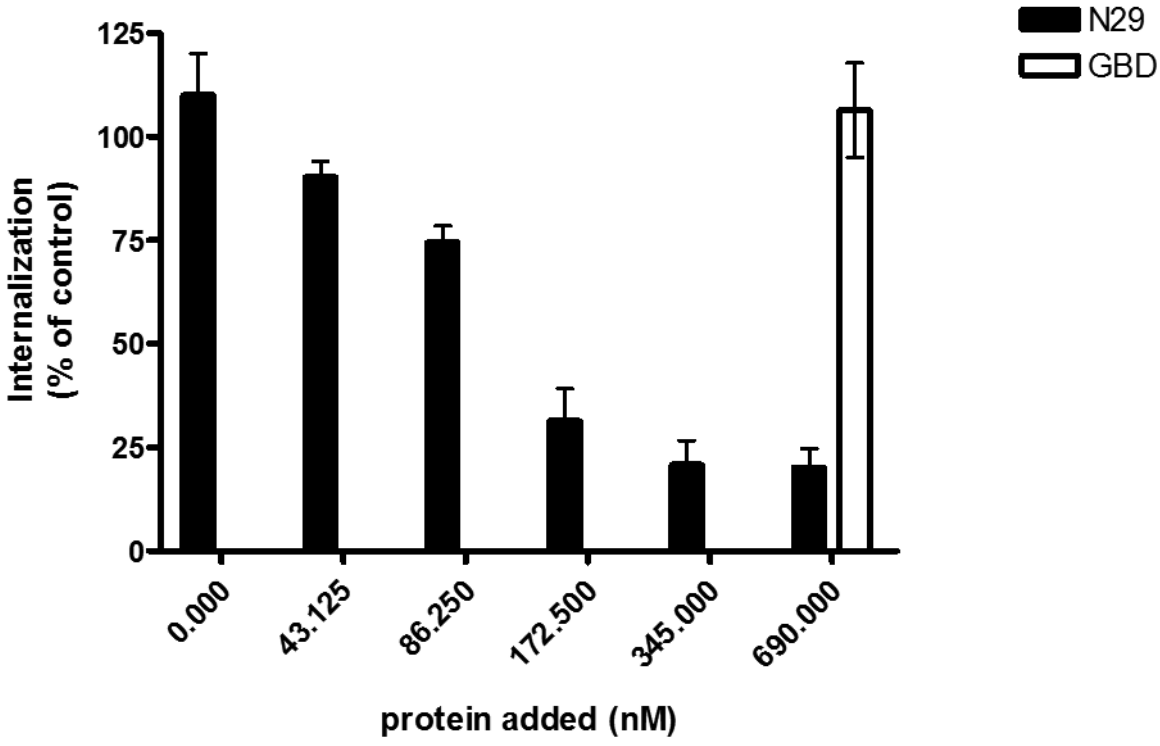
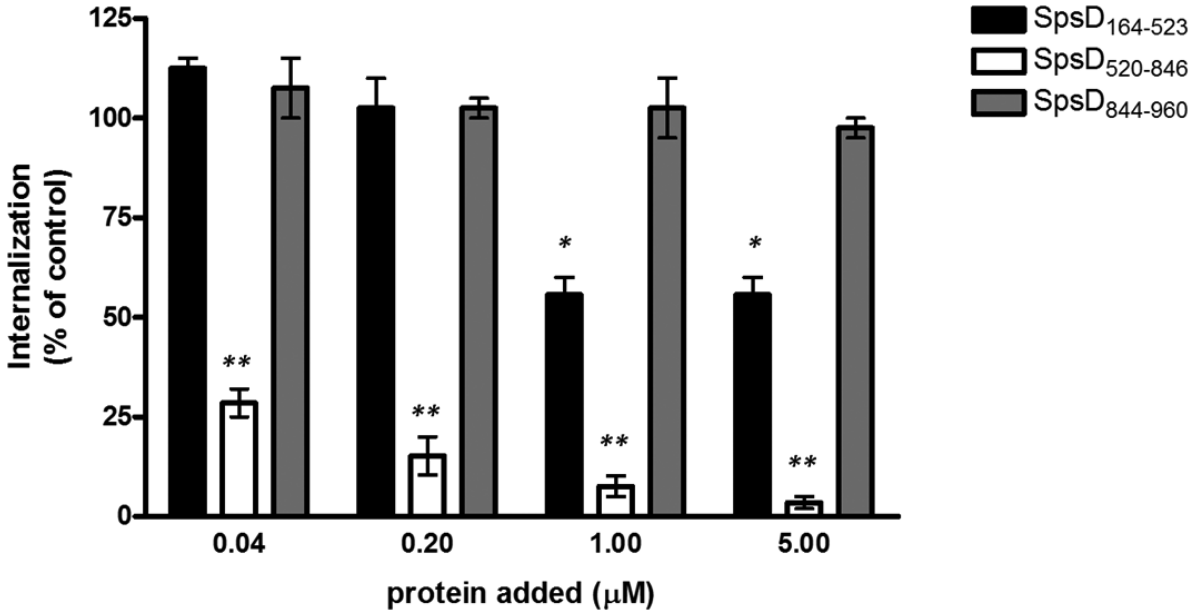


A



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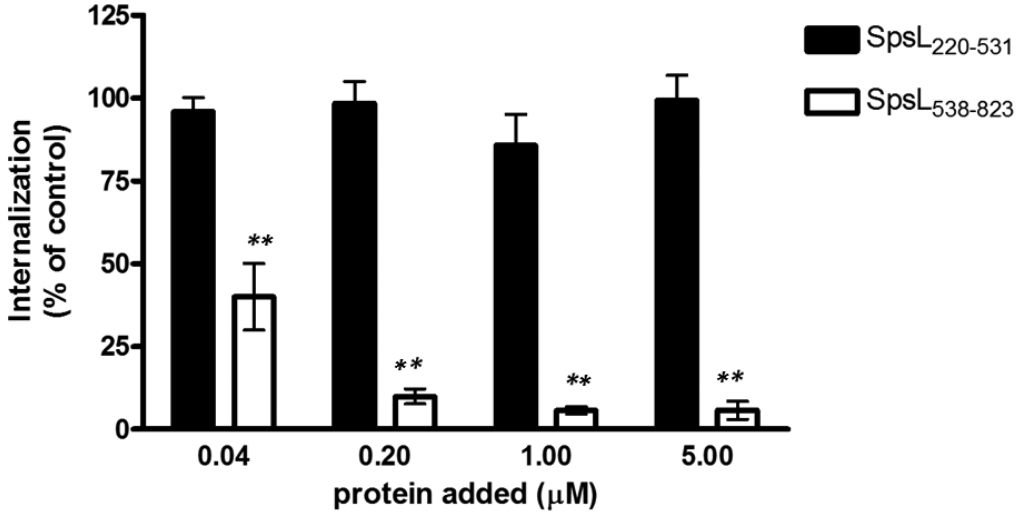

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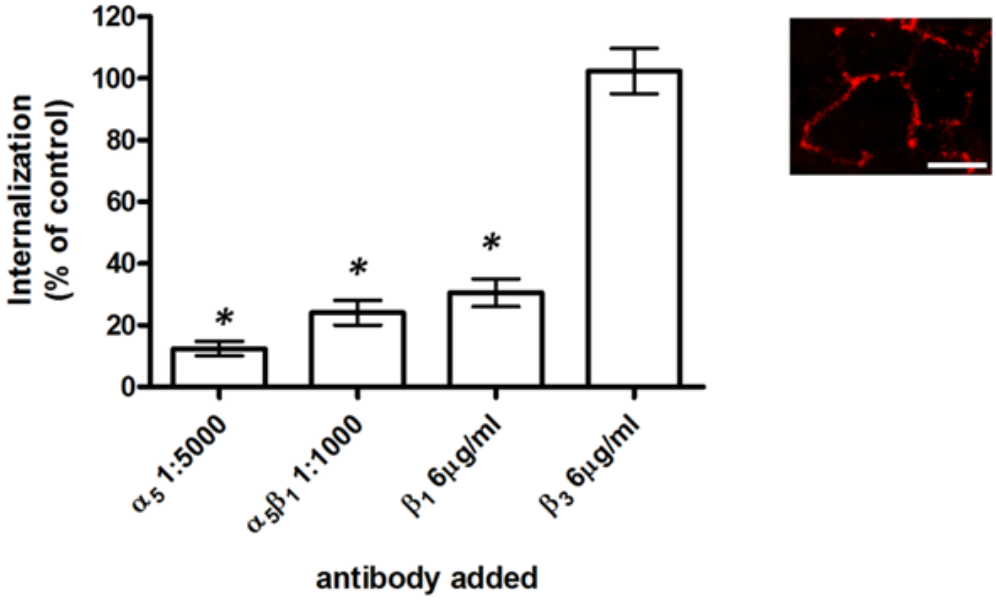

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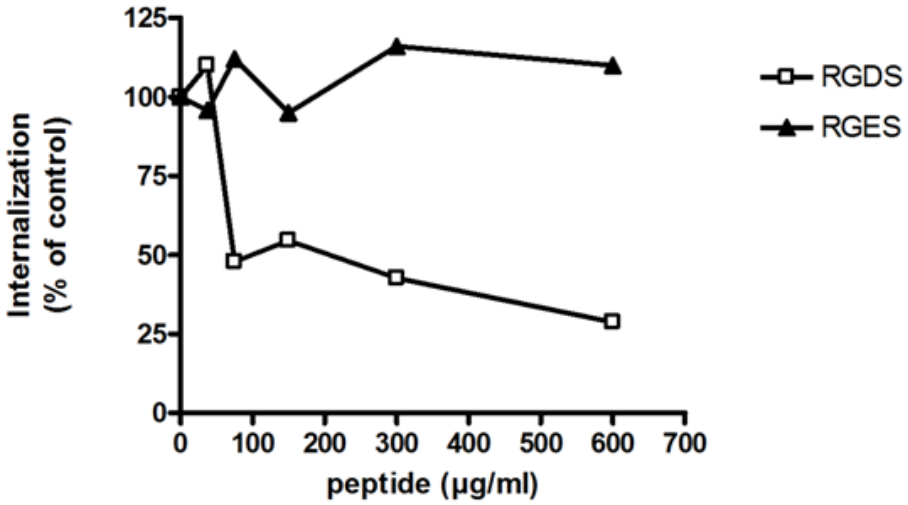

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